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Article

Terpenoids, Flavonoids and other Constituents of *Eupatorium betonicaeforme* (Asteraceae)

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Um novo diterpeno kaureno, caracterizado como ácido 15α-decanoiloxi-kaur-16-en-19-óico, juntamente com os compostos conhecidos: ácido pentacosanóico, 24α-etil-5α-colesta-7,22*E*-dien-3β-ol, ácido 15α-hidroxi-kaur-16-en-19-óico, 8β-angeloil-9β,10β-diidroxi-1-oxo-germacra-4*E*,11(13)dien-12,6α-olideo, 3β-hidroxiicosan-1,5β-olideo, acetate de taraxasterila, 7-O-metilcampferol, caempferol e nepetina foram isolados das flores de *Eupatorium betonicaeforme*. Adicionalmente, foram isolados das partes aéreas, o acetato de taraxasterila e a mistura binária de α- e β-amirina, enquanto foram obtidos das raízes, a mistura de β-sitosterol e estigmasterol, e 6-acetil-2,2-dimetilcroman-4-ona. A elucidação estrutural de todos os compostos isolados neste trabalho foi realizada por análise espectroscópica e comparação com dados descritos na literatura.

A new acylated kaurene diterpene, characterized as 15α-decanoyloxy-kaur-16-en-19-oic acid, along with nine known compounds: pentacosanoic acid, 24α-ethyl-5α-cholesta-7,22*E*-dien-3β-ol, 15α-hydroxy-kaur-16-en-19-oic acid, 8β-angeloyloxy-9β,10β-dihydroxy-1-oxo-germacra-4*E*,11(13)dien-12,6α-olide, 3β-hydroxyeicosan-1,5β-olide, taraxasteryl acetate, 7-*O*-methylkaempferol, kaempferol, and nepetin were isolated from the flowers of *Eupatorium betonicaeforme* (Asteraceae). In addition, from the aerial parts were isolated taraxasteryl acetate and α- and β-amyrin, while the mixture of β-sitosterol and stigmasterol, and 6-acetyl-2,2-dimethylchroman-4-one were isolated from the roots. The structure elucidation of all compounds was performed by spectroscopic analysis and comparison with published data from literature.

Keywords: *Eupatorium betonicaeforme*, Asteraceae, triterpenes, diterpenes, flavonoids, lactones

Introduction

The genus *Eupatorium* (family Asteraceae, tribe Eupatorieae, subtribe Eupatoriinae), is a taxonomically complex group of species with distribution centers mainly in Europe, eastern Asia and North America.[1] Several studies on *Eupatorium* species have been reported, revealing a great number and diversity of secondary metabolites, where the sesquiterpenes lactones of the guaianolide,[2] germacranolide,[3] heliangolide,[4] and eudesmanolide types[5] are the most characteristic compounds. Flavonoids,[6] diterpenes,[7] benzofurans,[8] pyrrolizidine alkaloids,[9] chromenes[10] and thymol derivatives[11] are also common. Triterpenes,[12] sesquiterpenes,[13] coumarins[14] and δ-lactones of the long chain type[15] have been occasionally isolated. A great number of *Eupatorium* species are employed in traditional medicine in the treatment of different pathologies. Indeed, a significant number of bioactive natural compounds have been reported from this renowned genus.[16-19]

As part of a continuing search on native plants from Northeast of Brazil, particularly of the Asteraceae family, the volatile oils from the roots and leaves of *E. betonicaeforme* yielded 2,2-dimethyl-6-vinylchroman-4-one (**1**) and 2-senecioyl-4-vinylphenol (**2**), the sesquiterpenes β-caryophyllene, γ-muurolene and bicyclogermacrene as the main constituents. Both oils showed larvicidal effects against *Aedes aegypti* larvae, particularly the oil from the roots.[20] The major constituents (**1** and **2**) were isolated and tested. Compound **1** was the most active compound and is probably responsible for the larvicidal effect.[20] Continuing with the phytochemical investigation of *E. betonicaeforme* this paper reports now its non-volatile chemical composition.

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Results and Discussion

The extract of the flowers of *E. betonicaeforme*, after successive column chromatography on Si gel, afforded the new secondary metabolite 15α-decanoyloxy-kaur-16-en-19-oic acid (**3**), together with the known compounds: pentacosanoic acid (**4**), 24α-ethyl-5α-cholesta-7,22*E*-dien-3β-ol (**5**),[21] 15α-hydroxy-kaur-16-en-19-oic acid (**6**),[22] 8β-angeloyloxy-9β,10β-dihydroxy-1-oxo-germacra-4*E*,11(13)dien-12,6α-olide (**7**),[23] 3β-hydroxyeicosan-1,5β-olide (**8**),[24] taraxasteryl acetate (**9**),[25] 7-*O*-methylkaempferol (**10**), kaempferol (**11**), and nepetin (**12**).[26] From the hexane extract of the aerial parts (leaves and stems) were isolated taraxasteryl acetate (**9**) and α- and β-amyrin (**13** and **14**),[25] while from the hexane extract of roots were obtained a mixture of β-sitosterol and stigmasterol (**15** and **16**) and 6-acetyl-2,2-dimethylchroman-4-one (**17**),[27] Figure 1. All of the known compounds were identified by comparison of their spectral data with those reported in the literature. Compound **7** was previously isolated from *Trichogonia salviaefolia* (Asteraceae) by Bohlmann *et al.*, and its molecular structure has been deduced by ^{1}H NMR and MS.[23] Complete ^{1}H and ^{13}C NMR chemical shifts of **7**, which were assigned by a combination of 1D and 2D NMR techniques, are listed in Table 1.

Compound **3** was isolated as a colorless waxy solid. The molecular formula $C_{30}H_{48}O_4$ was deduced by a combination of EIMS ([M]$^+$, *m/z* 472) and ^{13}C NMR data. The ^{13}C NMR spectrum showed a total of 30 carbon signals, among the characteristic signals at δ_C 29.9-29.3 and 14.3 consistent with the presence of aliphatic methylene groups of a fatty acyl moiety. The deshielded carbon signals at δ_C 184.8 and 174.1 indicated the presence of two carbonyl groups compatible with the presence of carboxylic acid and ester functions, respectively. IR absorption bands at 1730 and 1694 cm^{-1} further supported these findings. The additional deshielded carbon signals at δ_C at 155.7 and 110.0, as well the proton signal at δ_H 5.26 (2H, brs) clearly demonstrated the presence of an exomethylene moiety. In the HMBC spectrum were observed correlations for the exocyclic double-bond protons (δ_H 5.26) with the oxymethine carbon signal at δ_C 82.9 revealing the contiguity of these units. Comparison of the chemical shifts with the literature data indicated the presence of a kaur-16-en-19-oic acid bearing an oxygen atom at C-

Table 1. ^{1}H and ^{13}C NMR data for compounds **3** ($CDCl_3$) and **7** (acetone-d_6)

	3		**7**	
C	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)
1	37.8	2.15 (d, *J* 13.1)	216.6	-
2	19.2	1.44 (m) 1.60 (m)	39.1	3.64 (td, *J* 13.6; 3.0) 2.25 (dt, *J* 13.6; 3.7)
3	34.9	1.44 (m)	36.8	2.95 (m) 2.15 (dt, *J* 11.9; 3.0)
4	43.9	-	145.4	-
5	56.8	1.09 (d, *J* 11.9)	126.4	4.90 (br d, *J* 10.4)
6	20.9	1.86 (m) 1.77 (m)	76.3	5.21 (dd, *J* 7.8; 10.4)
7	40.7	1.86 (m)	50.4	2.83[c]
8	47.6	-	71.7	5.86 (dd, *J* 2.5; 4.0)
9	53.1	1.29-1.25	80.1	4.35 (dd, *J* 4.0; 6.4)
10	40.0	-	82.0	-
11	18.6	1.60 (m)	138.2	-
12	32.8	1.60 (m); 1.49	169.8	-
13	42.7	2.77 (br s)	120.4	6.11 (d, *J* 3.2)5.43 (d, *J* 3.2)
14	37.4	1.96 (d, *J* 11.5); 1.44	26.1	1.48 (s)
15	82.9	5.26 (s)	19.4	1.87 (d, *J* 1.3)
16	155.7	-	-	-
17	110.0	5.07 (s)	-	-
18	29.0	1.23 (s)	-	-
19	184.8	-	-	-
20	15.9	0.95 (s)	-	-
1’	174.1	-	167.1	-
2’	34.9	2.91 (t, *J* 7.5)	128.7	-
3’	25.3	1.60 (m)	139.2	6.13 (m)
4’			16.1	1.93 (dq, *J* 1.4; 7.3)
5’			20.8	1.82 (quint. *J* 1.4)
6’	29.9-29.3[a]	1.29-1.25 (m)[b]	-	-
7’			-	-
8’	32.0		-	-
9’	22.8		-	-
10’	14.3	0.87 (t, *J* 6.4)	-	-

[a] Chemical shifts for C-4’ - C-7’; [b] Chemical shifts for H-4’ - H-9’; [c] Multiplicity not determined due partial overlapping with the water peak.

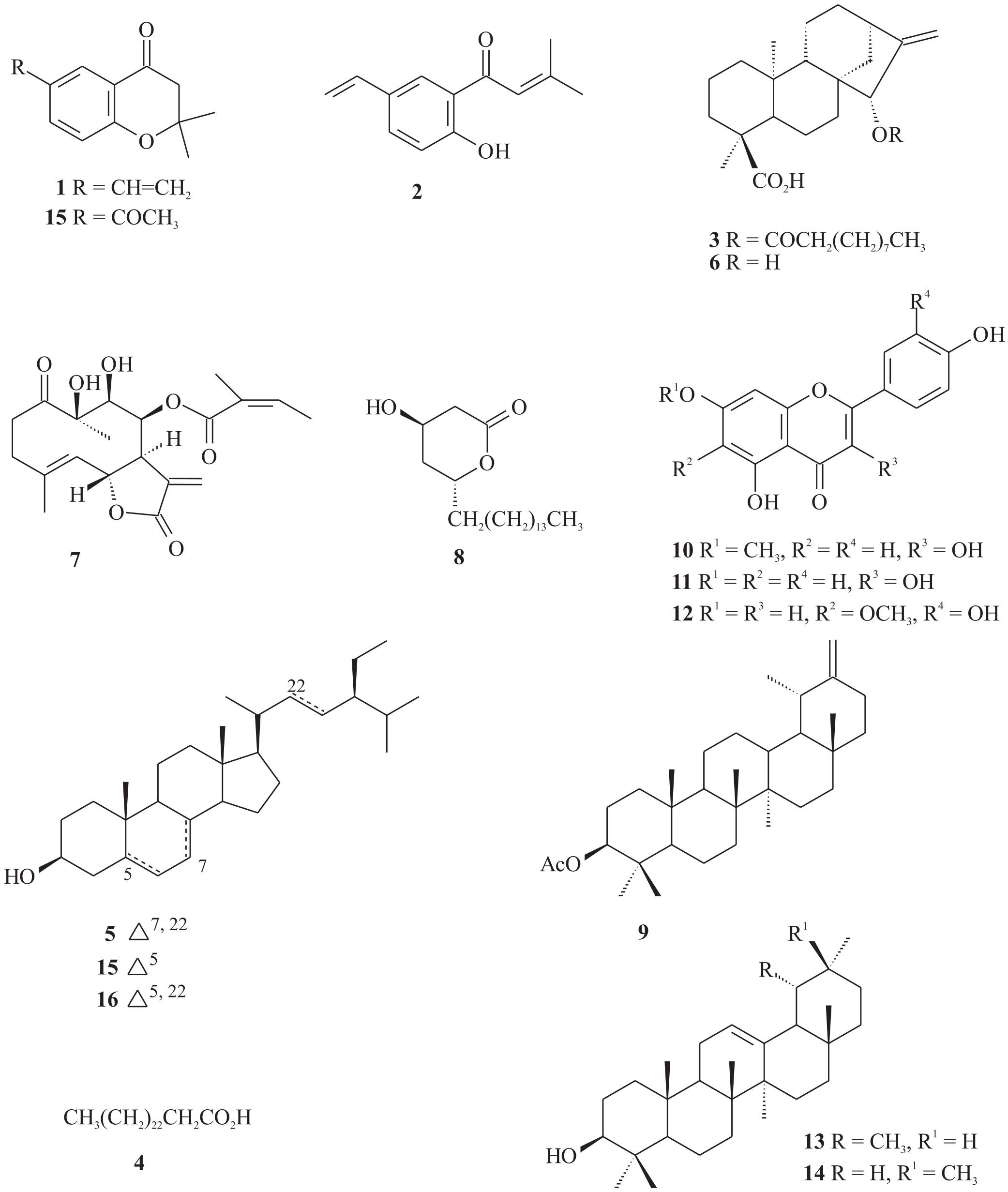


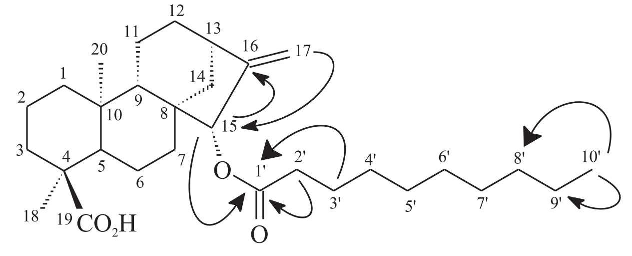
Figure 1. Chemical constituents isolated from *E. betonicaeforme*.

15.[22,28] This fact was also reinforced by the EIMS, which showed an intense ion peak at m/z 318 ($C_{20}H_{30}O_3$) related to a proton rearrangment followed by loss of the fatty side chain. In accordance with published data, it was assumed that diterpene **3** belongs to the *ent*-series, like the other kaurenes previously isolated from *Eupatorium*.[28,29] Additional support for this affirmation was the small negative specific rotation ($[\alpha]^{20}_{D}$ -0.66°, *c* 0.05, $CHCl_3$), which is characteristic of *ent*-kaurenes.[30] The location of the acyl moiety was supported by the long-range correlation of the oxymethine proton at δ_H 5.26 (H-15) with the ester carbonyl signal at δ_C 174.1, while the α-orientation of this unit was inferred by comparison of the ^{13}C NMR data with those from cinannamoylgrandifloric acid.[22] Thus, the structure of **3**, a new *ent*-kaurene derivative, was established as 15α-decanoyloxy-16-kauren-19-oic acid. Although the labdanes are the most frequently encountered diterpenes in plants of the genus *Eupatorium*, *ent*-kauranes are not uncommon.[28,29]

Experimental

General experimental procedures

Melting points were determined using a digital Mettler Toledo FP90 apparatus. The optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR spectra were



3

Figure 2. Selected HMBC correlations for compound **3**.

recorded using a Perkin-Elmer FT-IR 1000 spectrometer. EIMS was acquired with the direct insertion probe on a GC-MS Shimadzu spectrometer at 70 eV. NMR spectra were recorded on a Bruker Avance DRX-500 (500 MHz for ^1H and 125 MHz for ^{13}C) or DPX-300 (300 MHz for ^1H and 75 MHz for ^{13}C) spectrometers. Chemical shifts, given on the δ scale, were referenced to the residual undeuterated portion of the deuterated organic solvent, for proton [$(CD_3)_2CO$: δ_H 2.05; $CDCl_3$: δ_H 7.27], and the center peak of the deuterated solvent [$(CD_3)_2CO$: δ_C 206.68, 29.92; $CDCl_3$: δ_C 77.23]. Column chromatography was run using silica gel 60 (70 - 230 mesh, VETEC) and silica gel 60 (0.063-0.200 mm, MERCK). TLC was performed on precoated silica gel polyester sheets (Kieselgel 60 F_{254}, 0.20 mm, Merck) by detection with a spray reagent of vanillin/perchloric acid/EtOH solution followed by heating at 120 °C.

Plant material

Eupatorium betonicaeforme in the flowering stage was collected in May 2002, from Acarape County, Ceará, and identified by Prof. Elnatan B. Souza of the Universidade Estadual Vale do Acaraú - Ceará. Voucher specimen (# 29.104) has been deposited at the Herbario Prisco Bezerra (EAC) of the Departamento de Biologia, Universidade Federal do Ceará.

Extraction and isolation

Air-dried and powdered *E. betonicaeforme* parts were extracted with hexane: flowers (0.8 kg, 2 × 4 L), aerial parts (2.5 kg, 2 × 9 L) and roots (0.7 kg, 2 × 3 L), followed by EtOH with same amounts of solvent. After evaporation of the solvents under reduced pressure afforded the crude hexane and EtOH extracts, respectively: flowers (15.9 g and 82.9 g), aerial parts (51.6 g and 75.9 g), and roots (4.6 g and 18.0 g). The hexane extract from the flowers (15.9 g) was fractioned over Si gel to yield the follow fractions: hexane (0.4 g), hexane/EtOAc 8:2 (14.0 g), hexane/EtOAc 1:1 (0.8 g) and EtOAc (0.3 g). The hexane/EtOAc fraction 8:2 (14.0 g) was chomatographed over Si gel, eluting with hexane/EtOAc (9:1, 1:1), EtOAc, and MeOH to afford four fractions (F1-F4). F4 (8.7 g, hexane/EtOAc 9:1) was subjected to successive Si gel flash column chromatography using hexane/EtOAc 9.5:0.5 to yield **3** (80.0 mg) and pentacosanoic acid **4** (12.0 mg). Similarly, F2 (1.7 g, hexane/EtOAc 1:1) was subjected to repeated Si gel flash column chromatography using hexane/EtOAc 9:1 mixture to yield **5** (13.0 mg) and **6** (5.8 mg). The hexane/EtOAc fraction 1:1 (0.8 g) was subjected to Si gel flash column chromatography using $CHCl_3$/EtOAc 9:1 to give 145 subfractions of 10 mL each. **7** (16.0 mg) was isolated from fractions 65-72, while **8** (21.0 mg) was obtained from fractions 89-105. The EtOH extract of the flowers (82.0 g) was suspended in MeOH/H_2O 3:1 and partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH. The CH_2Cl_2 extract was evaporated under reduced pressure yielding a residue (24.0 g), which was fractioned over Si gel and eluted with hexane/EtOAc (7.5:2.5, 1:1, 2.5:7.5) and EtOAc. The hexane/EtOAc (7.5:2.5) fraction (4.0 g) was chromatographed over Si gel eluting with hexane/EtOAc (9:1, 8:2, 7:3, 1:1), EtOAc and MeOH to afford seven fractions (F1-F7). F2 (1.2 g) was subjected to Si gel flash column chromatography using hexane/EtOAc (9.9:0.1) to afford **9** (40.0 mg), while from fraction F6 (0.1 g), using the same chromatographic procedure and the solvent system hexane/EtOAc (7:3) was isolated **10** (3.0 mg). The hexane/EtOAc (1:1) fraction (1.1 g) was subjected to Si gel flash column chromatography eluting with a $CHCl_3$/EtOAc (8:2) gradient to yield **11** (4.2 mg) and **12** (370.0 mg). The hexane extract from the aerial parts (51.0 g) was fractioned over Si gel eluting with hexane, $CHCl_3$ and EtOAc. The hexane fraction (25.0 g) was chromatographed over Si gel using petroleum ether, CH_2Cl_2 and EtOAc as eluent. An aliquot of the petroleum ether fraction (1.5 g) was subjected to hydrolysis with KOH solution to yield a free fatty acids mixture (0.07 g) and an unsaponifiable fraction (0.7 g). Methylation of the fatty acids with MeOH/HCl (1.0%) followed by GC/MS analysis of the methyl esters mixture allowed the identification of: hexadecanoic (65.0%), heptadecanoic (1.2%), octadecanoic (7.3%), eicosanoic (3.5%), docosanoic (9.0%), tricosanoic (1.0%), and tetracosanoic acids (3.5%). TLC analysis indicated that the CH_2Cl_2 fraction (9.8 g) was rich in compound **9**, which was recrystallized in acetone to afford taraxasteryl acetate (736.0 mg). The CH_2Cl_2 fraction (21.0 g) after successive Si gel column chromatography using hexane and increasing amounts of EtOAc afforded a binary mixture of **13** and **14** (7.0

mg). The hexane fraction from the roots (4.6 g) was fractioned over Si gel eluting with hexane, hexane/CH_2Cl_2 1:1, CH_2Cl_2 and EtOAc. From the fraction hexane/CH_2Cl_2 (1:1) fraction (1.6 g) was obtained a mixture of **15** and **16** (30.0 mg). The EtOH extract was fractioned over Si gel eluting with hexane/EtOAc (8:2, 6:4, 4:6, 2:8), EtOAc and MeOH. The hexane/EtOAc (8:2) fraction (2.7 g) was subjected to successive Si gel column chromatography using increasing amounts of EtOAc in hexane to yield **17** (42.0 mg).

*15α-Decanoyloxy-kaur-16-en-19-oic acid (**3**).* Colorless waxy solid; $[\alpha]_D^{20}$ -0.66° (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max}/cm^{-1}: 3350-2700, 1730, 1694, 1464, 1242, 1165; EIMS (70 eV) *m/z* 472 $[M]^+$ (5), 318 (25), 300 (80), 285 (90), 255 (50), 104 (65), 91 (45), 71 (82), 57 (100); ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data, Table 1.

*8β-Angeloyl-9β,10β-dihydroxy-1-oxo-germacra-4E,11(13)dien-12,6α-olide (**7**).* Colorless needles; mp 164-165 °C; $[\alpha]_D^{20}$-0.24° (*c* 0.05, acetone); IR (KBr) ν_{max}/cm^{-1}: 3437, 2929, 1736, 1699, 1154, 1107; EIMS *m/z* 378 $[M]^+$ (absent), 312 (10), 295 (5), 277 (15), 260 (12), 232 (20), 207 (12), 108 (13), 83 (100); ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data, Table 1.

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